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Experimental Hematology

Effects of radiation on survival and recovery of T lymphocyte subsets in C3H/HeN mice

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Abstract

The aims of this study were to determine the radiosensitivities of murine thymic and splenic $CD4^+$ and $CD8^+$ lymphocytes and to evaluate the regeneration of these cells in a model of radiation-induced hematopoietic and immune suppression. $CD4^+$ and $CD8^+$ cells were quantitated using two-color flow-cytometric analysis. Cells obtained from C3H/HeN mice 24 hours after exposure to 0.25–8.0 Gy (0.4 Gy/min) ^{60}Co were used to determine D_0 values. Thymic $CD4^+$ cells contained a radiosensitive subpopulation with a D_0 of 0.97 ± 0.05 Gy and a radioresistant subpopulation that survived exposures up to 8.0 Gy. $CD8^+$ cells also contained a radiosensitive subpopulation with a D_0 of 1.24 ± 0.05 Gy and a radioresistant subpopulation with a D_0 of 3.93 ± 2.01 Gy. Double-positive thymic $CD4^+/CD8^+$ cells were uniformly radiosensitive, with a D_0 of 1.03 ± 0.28 Gy. Multiple T lymphocyte subpopulations based on radiosensitivity and CD4/CD8 antigen expression were also observed in the spleen. When mice were exposed to a sublethal 6.5-Gy radiation dose and recovery of T lymphocyte subsets was monitored, the relative radioresistance of $CD4^+$ cells resulted in a selective enrichment of these cells among the surviving thymocytes and splenic lymphocytes. This relative enrichment of $CD4^+$ cells became even more prominent 7 days after irradiation, when atrophy of the organs was greatest. Similar, although less dramatic, effects were observed for $CD8^+$ cells. These studies demonstrate that (1) multiple T lymphocyte subpopulations can be identified based on radiosensitivity and CD4/CD8 antigen expression; (2) both $CD4^+$ and $CD8^+$ cells contain radioresistant subpopulations, with the $CD4^+$ subpopulation being more resistant than the $CD8^+$ subpopulation; and (3) although the number of radioresistant $CD4^+$ cells is quite small, they persist in increased proportions during the periods preceding and corresponding to postirradiation hematopoietic recovery.

Key words: T lymphocytes—Radioresistance—Hematopoiesis

Introduction

Substantial hematopoietic recovery following chemotherapy or radiotherapy requires stem cells to proliferate and differentiate into specific progenitor cells capable of giving rise to functional mature cells. Following sublethal chemotherapy or radiotherapy regimens, hematopoietic recovery will ultimately ensue from surviving endogenous hematopoietic stem and progenitor cells. Following more intense suppressive regimens, however, recovery can be facilitated only by transplanting bone marrow cells from a suitable donor [1].

Hematopoietic recovery in mice given bone marrow transplants has been demonstrated to be enhanced by supplementing donor bone marrow cells with normal syngeneic thymic lymphocytes [2]. Since the cells responsible for this improvement were eliminated by in vitro treatment with an anti-Thy-1 serum and complement, they were subsequently named "anti-theta-sensitive regulatory cells" (TSRC) [3]. These cells were also found in the spleen and bone marrow. Later, in vitro studies of erythropoiesis revealed that TSRC actually consisted of two distinct $Thy\text{-}1^+$ subpopulations; one that enhanced hematopoietic recovery and another that suppressed it [4]. The two TSRC subpopulations differed in a number of criteria [4–8]. For example, TSRC that enhanced hematopoietic recovery were resistant to cyclophosphamide and radiation, while TSRC that suppressed recovery were sensitive to these agents [6]. The fact that helper TSRC appeared to be relatively radioresistant suggested that a portion of these cells may persist in irradiated animals and play a role in facilitating hematopoietic recovery, even following radiation exposures such as those used in bone marrow transplant preparation regimens [2].

Although murine lymphocytes have generally been reported to have a D_0 of less than 2 Gy [1], it has been known for years that a small population of radioresistant $Thy\text{-}1^+$ lymphocytes survive in vivo in heavily irradiated animals (for a review see Anderson and Warner [9]). Cell transfer studies indicate that these radioresistant T lymphocytes function as helper cells in reconstituting immune responses. Suppressor function, on the other hand, is abrogated by exposure to radiation. This differential sensitivity to irradiation between helper and suppressor lymphocytes, as well as similar differences in sensitivity to cyclophosphamide [6], seems to mirror the differential sensitivity patterns to these agents found in helper and suppressor TSRC. Thus, it is tempting to hypothesize that the helper and suppressor cells of the TSRC system may be identical to helper and suppressor T lymphocytes of the immunoregulatory system.

It is now known that helper T lymphocyte function is associated primarily with cells that express the CD4 antigen [10], while suppressor T lymphocytes express the CD8 antigen [11]. However, $CD4^+$ and $CD8^+$ T lymphocytes have not been well characterized with regard to their radiosensitivities and persistence in vivo after irradiation. To better understand the relationship between TSRC and immunoregulatory T lymphocytes, we examined the radiosensitivity of thymic and splenic lymphocyte subsets defined by CD4 and CD8

antigens. Furthermore, we followed changes in these cell populations concomitant with hematopoietic regeneration in sublethally irradiated mice. Our results show that both the thymus and the spleen contain a subpopulation of extremely radioresistant $CD4^+$ cells. As a result of the radioresistance of these cells vis-à-vis the radiosensitivity of other lymphoid cells, irradiated animals become proportionately enriched with $CD4^+$ cells. Although the number of $CD4^+$ cells is quite small, the enrichment persists during the early critical phases of postirradiation hematopoietic regeneration.

Materials and methods

Mice

C3H/HeN female mice (~20 g) were purchased from Charles River Laboratories (Wilmington, MA). Mice were maintained in a facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) in Micro-Isolator cages on hardwood-chip contact bedding and were provided commercial rodent chow and acidified water (pH 2.5) ad libitum. Animal rooms were equipped with full spectrum light from 6 a.m. to 6 p.m. and were maintained at 21°C ± 1°C with 50 ± 10% relative humidity using at least 10 air changes/h of 100% conditioned fresh air. On arrival, all mice were tested for *Pseudomonas* and quarantined until test results were obtained. Only healthy mice were released for experimentation. All animal experiments were approved by the Institute Animal Care and Use Committee before performance.

Irradiation

Mice were placed in ventilated Plexiglas boxes and exposed bilaterally to gamma radiation from the AFRRI ^{60}Co source. Prior to animal irradiations, the midline tissue (MLT) dose rate was measured by placing a 0.5-cc tissue equivalent ionization chamber (calibration factor traceable to the National Institute of Standards and Technology) at the center of a cylindrical acrylic mouse phantom (2.5-cm diameter). The tissue-to-air ratio (TAR) for this array, defined as the ratio of the dose rate in free air to the dose rate measured in the phantom, was 0.96. Exposure time was adjusted so that each animal received from 0.25 to 8.0 Gy MLT at a dose rate of 0.4 Gy/min. Variation within the exposure field was less than 3%. The techniques used for these measurements were in accordance with the American Association of Physicists in Medicine protocol for the determination of absorbed dose from high-energy photon and electron beams [12].

Cell suspensions

The cell suspensions for each assay represented tissues from three animals. Animals were killed by cervical dislocation, and the thymuses and spleens were aseptically excised. Cells from the organs were pressed through stainless steel mesh screens and collected in McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum. Single-cell suspensions were prepared by repeatedly passing the cells through a 20-gauge needle. Erythrocytes were removed by lysis with hypotonic ammonium chloride, and nucleated cells were resuspended in Hanks' balanced salt solution, without calcium and magnesium, containing 1% fetal bovine serum (HBSS + 1% FBS). The number of nucleated cells in the suspension was determined by a Coulter counter. Cytocentrifuge preparations of the cell suspensions were prepared, stained with Diff-Quick, and used for differential cell counts.

Lymphocyte subset analysis

Cells were diluted to 1×10^7 cells/mL in HBSS + 1% FBS, and 100-µL portions were labeled by concurrent incubation with 5 µL each of phycoerythrin-conjugated anti-CD4 and fluorescein-isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibodies (Becton Dickinson, Mountain View, CA). The cells were incubated on ice for 30 minutes and washed once with HBSS + 1% FBS. Flow-cytometric analysis was performed using a Coulter EPICS V flow cytometer and the MDADS computer system. Gates were set on the forward-angle light scatter vs. 90-degree light scatter histograms to eliminate dead cells and debris. Two-color analysis was performed with the Quad-Stat program of the MDADS system. The number of cells of a particular subset in an organ was determined by multiplying the number of cells per organ, as determined by Coulter counts, by the proportion of cells with the given phenotype.

D_0 determinations

Mice were exposed to graded doses of radiation from 0.25 to 8.0 Gy, and tissues were collected 24 hours later. Cell suspensions were prepared and subpopulations analyzed by flow-cytometric analysis. The D_0 values were determined by plotting the surviving fraction (where surviving fraction = number of cells surviving after a given radiation dose/number of cells in nonirradiated controls) against the radiation dose. The best fit for linear portions of the curves and the Y-intercepts were calculated by nonlinear least-squares fit and the D_0, the radiation dose where the surviving fraction is equal to 0.37, was extrapolated from the exponential portion of the curves.

Spleen colony-forming unit (CFU-S) assay

CFU-S were evaluated by the method of Till and McCulloch [13]. Each colony has been shown to arise from the clonal proliferation of multipotent hematopoietic stem cells. Recipient mice were exposed to 9 Gy of total body irradiation to eradicate endogenous hematopoietic stem cells. After 3 to 5 hours, 5×10^4 bone marrow or 5×10^5 spleen cells were intravenously injected into the irradiated recipients. Twelve days after transplantation, the recipients were killed by cervical dislocation, and their spleens were removed. The spleens were fixed in Bouin's solution, and the grossly visible colonies were counted. For each experiment, groups of five mice were used, and the experiments were repeated twice.

Granulocyte-macrophage colony-forming cell (GM-CFC) assay

Hematopoietic progenitor cells committed to granulocyte and/or macrophage development were assayed using a double-layer agar GM-CFC assay [14]. Mouse endotoxin serum (5% vol/vol) was added to feeder layers as a source of colony stimulating factor. Colonies (>50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 5% CO_2. Triplicate plates were cultured for each cell suspension and experiments were repeated twice.

Results

Radiosensitivity of thymic and splenic T lymphocyte populations

$CD4^+$ thymocytes consisted of two subpopulations relative to radiosensitivity (Fig. 1A): a sensitive subpopulation with a D_0 of 0.97 ± 0.05 Gy and a resistant subpopulation that survived radiation exposures up to 8.0 Gy (the highest radiation dose evaluated), making it impossible to calculate the D_0 for this subpopulation. The radiosensitive thymic subpopulation contained approximately 82% of the $CD4^+$ cells, while the remaining 18% were in the radioresistant subpopulation. Thymic $CD8^+$ cells also consisted of two subpopulations, the first with a D_0 of 1.24 ± 0.05 Gy and the second with a D_0 of 3.93 ± 2.01 Gy (Fig. 1B). Thymic $CD4^+/CD8^+$ cells, the predominant thymocyte subset in normal animals, were uniformly radiosensitive, with a D_0 of 1.03 ± 0.28 Gy (Fig. 1C). Figure 1D shows

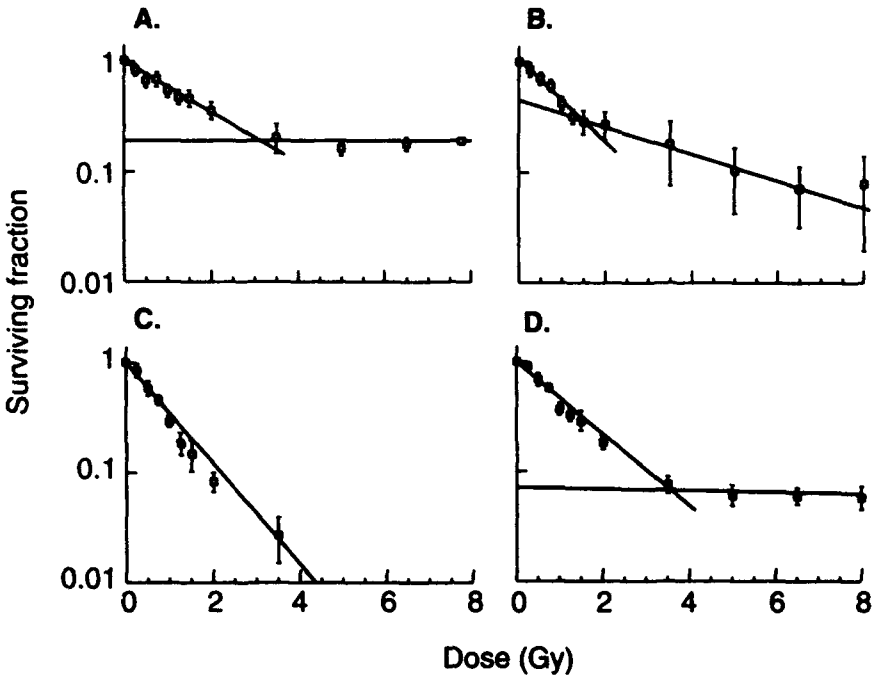

Fig. 1. Radiosensitivity of thymocyte subsets in C3H/HeN mice. Mice were exposed to graded doses of ^{60}Co radiation and thymocyte subset analysis was performed 24 hours later. **A.** $CD4^+$ cells. **B.** $CD8^+$ cells. **C.** $CD4^+/CD8^+$ cells. **D.** Total thymic cellularity. Data represent the mean ± 1 standard error of the mean (SEM) for two or more experiments with thymocytes pooled from three mice for each experiment. The surviving fraction is the ratio of the number of cells positive for the indicated antigens in irradiated animals to the number of cells positive for the indicated antigen in unirradiated controls.

that approximately 93% of the total thymocyte population was radiosensitive, with a D_0 of 1.14 ± 0.08 Gy.

Similar results were seen in the spleen, but precise D_0 values could not be determined because of high levels of autofluorescence in the spleen cells obtained from mice exposed to the higher doses of radiation.

Repopulation of thymic and splenic T lymphocyte populations in mice recovering from myeloablative radiation exposure

Total thymic cellularity at 24 hours after exposure decreased more than 15-fold, from 102.72×10^6 to 6.48×10^6 cells. Concomitant reductions in cell numbers occurred in each of the thymocyte subsets defined by the CD4 and CD8 antigens; however, the degree of cytoreduction varied among the different cell types based on their radiosensitivities (Table 1). $CD4^+/CD8^+$ cells, the predominant thymic cell type, decreased the most of any subset from 73.16×10^6 to 0.45×10^6 cells per thymus (0.6% survival). Single-positive cells were affected to a lesser degree. The number of $CD4^+$ thymocytes fell from 14.68×10^6 to 1.96×10^6 cells per thymus (13.4% survival) while the $CD8^+$ thymocytes decreased from 3.98×10^6 to 0.24×10^6 cells per thymus (6.0% survival). These values compared well with values calculated from the data in the D_0 experiments, which indicated that only 0.7% of the $CD4^+/CD8^+$, 18.5% of the $CD4^+$, and 8.4% of the $CD8^+$ thymocytes should remain 24 hours after a 6.5-Gy radiation exposure.

Large reductions both in total cellularity and in the number of cells in each specific subset also occurred in the spleen. In this organ, the $CD4^+$ cells declined from 14.94×10^6 to 5.92×10^6 cells per spleen (39.6% survival), while $CD8^+$ decreased from 5.41×10^6 to 0.31×10^6 cells per organ (5.7% survival). Double-positive T lymphocytes were not seen in the spleen.

Table 1. Residual T lymphocyte subsets in C3H/HeN thymus and spleen 24 hours after exposure to 6.5 Gy ^{60}Co radiation

Cell type	Control	6.5 Gy
	Thymic cellularity ($\times10^6$)	
Total	102.72±3.13	6.48±0.90
$CD4^+/CD8^+$	73.16±5.85	0.45±0.06
$CD4^+$	14.68±1.70	1.96±0.40
$CD8^+$	3.98±0.43	0.24±0.06
$CD4^-/CD8^-$	10.55±1.52	3.74±0.83
	Splenic cellularity ($\times10^6$)	
Total	97.86±10.80	16.29±6.56
$CD4^+$	14.94±2.63	5.92±4.54
$CD8^+$	5.41±1.09	0.31±0.22
$CD4^-/CD8^-$	74.41±7.91	9.95±1.73

The differences in cytoreduction postirradiation among the various subsets resulted in selective enrichment of $CD4^+$ cells. Specifically, the relative proportion of $CD4^+$ cells in the thymus increased from 15.3 to 30.0% of the total remaining cells, and in the spleen they increased from 14.3 to 33.4%. On the other hand, the percentage of $CD8^+$ cells remained constant at about 3.8% in the thymus and decreased from 5.5 to 1.9% in the spleen. The proportion of double-positive cells in the thymus also decreased dramatically from 71.2 to 0.5%.

The radiation-induced atrophy of the thymus was still evident 7 days after irradiation, and the number of cells in each of the thymocyte subsets remained lower than in controls. At that time, there were $0.76 \pm 0.29\times10^6$ $CD4^+$ cells, 0.29 ±

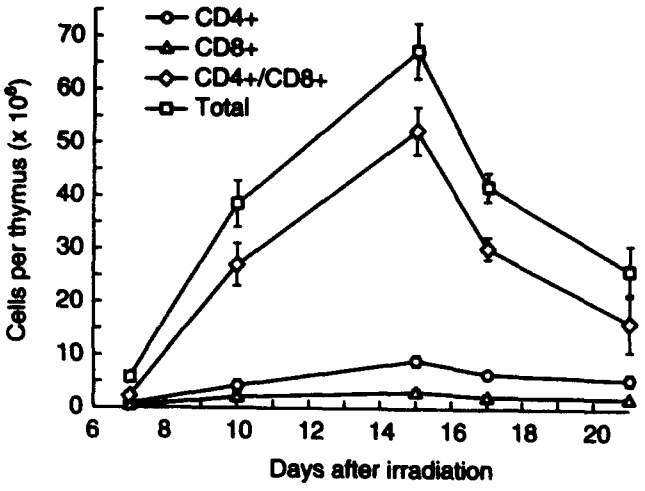

Fig. 2. Recovery of thymocyte subsets after sublethal irradiation. Mice were exposed to 6.5 Gy of ^{60}Co radiation, and the thymuses were harvested at the time points indicated. Thymocyte subset analysis was performed as in Figure 1. Data represent the mean ± 1 SEM for three or more experiments with thymocytes pooled from three mice for each experiment. $CD4^+$ cellularity in nonirradiated control mice was $14.68 \pm 1.70 \times 10^6$ cells per thymus; $CD8^+$ cellularity was $3.98 \pm 0.43 \times 10^5$ cells per thymus; and $CD4^+/CD8^+$ cellularity was $73.16 \pm 5.85 \times 10^6$ cells per thymus.

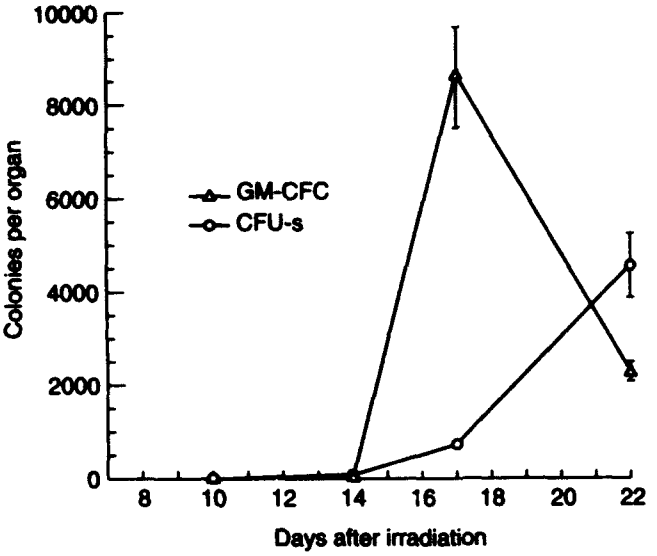

Fig. 4. Recovery of CFU-S and GM-CFC in irradiated C3H/HeN mice. Mice were exposed to 6.5 Gy of ^{60}Co radiation and splenic CFU-S and GM-CFC contents were determined at the time points indicated. Data represent the mean ± 1 SEM of pooled values obtained from two separate experiments. In normal nonirradiated controls, CFU-S content was $7.3 \pm 0.7 \times 10^3$ and GM-CFC content was $4.4 \pm 0.5 \times 10^3$.

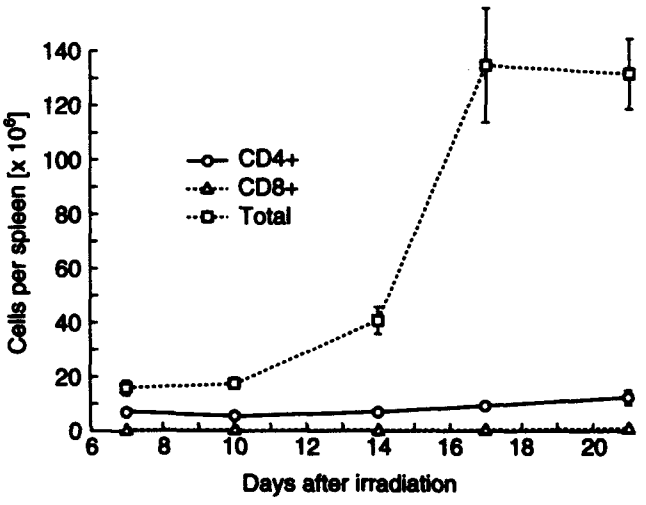

Fig. 3. Recovery of T lymphocyte subsets in the spleen after sublethal irradiation. Mice were exposed to 6.5 Gy of ^{60}Co radiation and the spleens were harvested at the time points indicated. Spleen subset analysis was performed as in Fig. 1. Data represent the mean ± 1 SEM for three or more experiments with cells pooled from three mice for each experiment. $CD4^+$ cellularity in nonirradiated control mice was $14.94 \pm 2.63 \times 10^6$ cells per spleen, and $CD8^+$ cellularity was $5.41 \pm 1.09 \times 10^6$ cells per spleen.

0.09×10^6 $CD8^+$ cells, and $2.30 \pm 0.82 \times 10^6$ $CD4^+/CD8^+$ cells per thymus. Cellular regeneration was evident at 10 days, peaked at 14 days, and returned toward normal thereafter (Fig. 2). Double-positive $CD4^+/CD8^+$ cells made up the bulk of the regenerating thymocytes. $CD4^+$ cells and $CD8^+$ cells followed a similar cycle of regrowth but with slower kinetics.

In the spleen, total cellularity remained low for 10 days and then began to increase between 10 and 14 days after irradiation (Fig. 3). The number of spleen cells increased rapidly to a peak value at day 17 postirradiation and remained elevated through the end of the experimental observation period. Splenic $CD4^+$ cells remained low through the first 10 days and then began to slowly increase until the end of the experiment. The $CD8^+$ cells, which were reduced 3.5-fold by irradiation, remained at very low levels throughout the observation period. Concomitant with these studies evaluating T lymphocyte subset recovery, splenic CFU-S and GM-CFC recovery were also monitored in mice exposed to 6.5 Gy ^{60}Co radiation (Fig. 4). CFU-S and GM-CFC numbers decreased to undetectable levels for at least 7 days and then recovered rapidly. By day 22 postirradiation, splenic CFU-S recovery was approximately 65% of normal controls, and GM-CFC recovery was actually 180% of normal controls as early as 17 days postirradiation.

Effect of radiation on survival and repopulation of non-T lymphocytes in the thymus and spleen

Inherent in these studies was also the opportunity to observe the radiation-induced changes in non-T lymphocytes in the thymus and spleen, that is, the cells staining negative for CD4 and CD8. These cells consisted of a mixture of macrophages, natural killer cells, progenitor cells, and, in the spleen, B lymphocytes. The radiosensitivity of the $CD4^-/CD8^-$ cell popula-

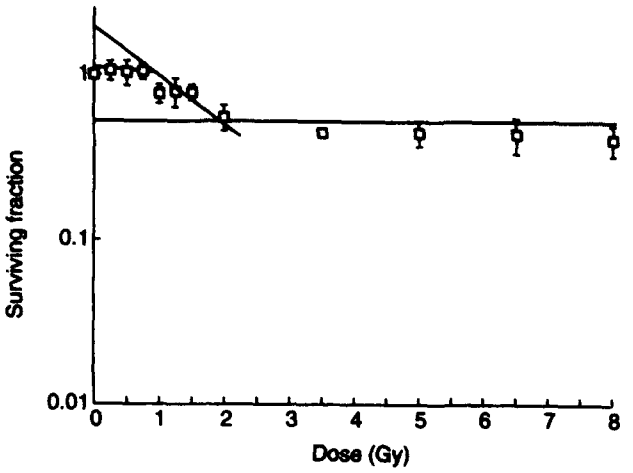

Fig. 5. Radiosensitivity of $CD4^-/CD8^-$ thymocytes in C3H/HeN mice. Mice were exposed to graded doses of ^{60}Co radiation, and thymocyte subset analysis was performed 24 hours later. Data represent the mean ± 1 SEM for two or more experiments with thymocytes pooled from three mice for each experiment. The surviving fraction is the ratio of the number of cells positive for the indicated antigens in irradiated animals to the number of cells positive for the indicated antigens in unirradiated controls.

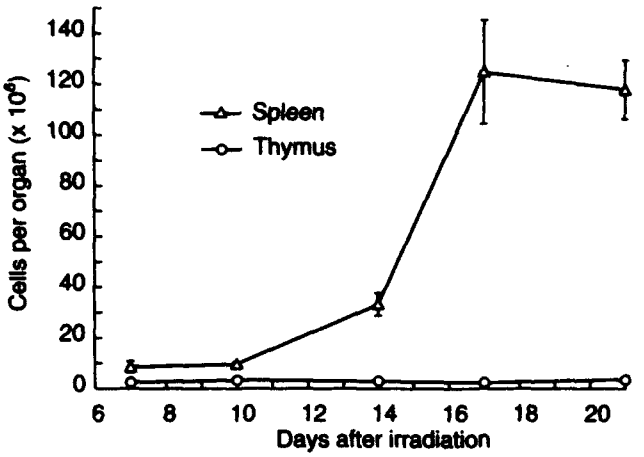

Fig. 6. Recovery of thymic and splenic $CD4^-/CD8^-$ cells after sublethal irradiation. Recovery of the $CD4^-/CD8^-$ thymocytes and splenic lymphocytes was determined from the experiments described in Figures 2 and 3, respectively. $CD4^-/CD8^-$ cellularity in nonirradiated control mice was $10.55 \pm 1.52 \times 10^6$ cells per thymus and $74.41 \pm 7.91 \times 10^6$ cells per spleen.

tion in the thymus is illustrated in Figure 5. These cells contained a radiosensitive subpopulation with a D_0 of 2.28 ± 0.74 Gy and an n of 1.37 ± 0.26. In addition, these cells contained a radioresistant subpopulation with a D_0 greater than 8 Gy. Recovery of the $CD4^-/CD8^-$ cells following a sublethal 6.5-Gy irradiation is illustrated in Figure 6. In the thymus, the number of $CD4^-/CD8^-$ cells remained relatively constant throughout the 21-day postirradiation period, while in the spleen, the observed changes in cellularity during recovery were primarily driven by changes in the number of double-negative cells.

Discussion

Our results indicate that the radiosensitivities of the $CD4^+$ and $CD8^+$ T lymphocytes are comparable, respectively, to the radiosensitivities of helper and suppressor TSRC reported by Sharkis et al. [6]. The $CD4^+$ cells that we observed in the thymuses of irradiated animals exhibited properties characteristic of the TSRC that stimulate hematopoietic recovery. Helper TSRC, which make up less than 10% of the cells in normal thymuses, are reported to be radioresistant [5]. Although, in our studies, the total number of thymus cells was drastically reduced 24 hours after irradiation, radioresistant $CD4^+$ cells were easily found in the thymuses of irradiated animals (Table 1). These results support Huiskamp and van Ewijk's immunocytochemistry studies [15], in which they too observed radioresistant $CD4^+$ cells in the thymuses of mice 24 hours after irradiation. Our use of two-color analysis to simultaneously test for CD4 and CD8 antigen expression confirmed the single-positive phenotype of the radioresistant $CD4^+$ cells, a characteristic typical of mature T lymphocytes in the thymic medulla and the peripheral organs [16]. Exposure of mice to 6.5 Gy radiation left an average of 1.96×10^6 radioresistant $CD4^+$ cells in the thymuses 24 hours after irradiation (Table 1). This corresponds to approximately 2% of the thymocyte population in unirradiated animals and compares well with the estimate of Sharkis et al. [5] for the size of the stimulatory TSRC population.

The D_0 determinations (Fig. 1) show that the surviving fraction of $CD4^+$ cells at radiation doses of 4 to 8 Gy is approximately 0.18. However, the remainder of the $CD4^+$ cells has a D_0 of 0.97 Gy, which is comparable to the D_0 of lymphocytes in general [1]. Radiation-induced apoptosis is the most likely cause of death of the radiosensitive cells, since resting lymphocytes are known to be extremely sensitive to this process while mitogen-activated lymphocytes are known to be resistant to this form of cell death [17]. The mechanisms responsible for this are unknown but may be related, in part, to the cell cycle. It is well known that cells in late S-phase of DNA synthesis are more radioresistant than cells in other phases of the cell cycle [18]. This increase in radioresistance is mediated by DNA repair and results in an increase in the n value and a decrease in the slope of the survival curves in the D_0 studies [19]. However, the percent of $CD4^+$ cells in S-phase is only 5 to 7% [20], which does not account for the 18% survival of $CD4^+$ cells reported here.

Sharkis et al. [5] have shown that helper TSRC that survive an initial radiation exposure appear to persist in irradiated animals. Likewise, radioresistant $CD4^+$ cells detected in our studies appear to persist in irradiated animals, since these cells were still present on each day tested between 7 and 21 days postirradiation (Fig. 2). Huiskamp and van Ewijk [15] reported that radioresistant $CD4^+$ thymocytes could be seen as late as 10 days after irradiation. However, due to the qualitative nature of their immunocytochemistry techniques, they were unable to provide a quantitative estimate of the size of this population. Using quantitative techniques based on flow cytometry, our data shows that the number of $CD4^+$ cells decreased between days 1 and 7 after irradiation, but even at the nadir of thymic cellularity (5.8×10^6 cells per thymus) approximately 7.6×10^5 $CD4^+$ cells could be detected.

Tomooka et al. [21] have also examined the changes in thymocyte subsets after irradiation using techniques very similar to those we used in this report. Their results are comparable to ours, but their interpretation differs significantly with respect to the persistence of the $CD4^+$ cells in the thymus. They report an absence of mature thymocytes at day 7 postirradiation; however, even after exposure to 8 Gy ^{60}Co radiation, a population of phenotypically mature $CD4^+$ cells is evident at each time point depicted in their flow-cytometric data. Taken together, the data presented in our studies and the data from the reports cited above support the postulate that a small population of $CD4^+$ cells persists in the thymuses of irradiated mice and may contribute to thymic as well as hematopoietic regeneration.

In the thymus, most $CD8^+$ cells were sensitive to the doses of radiation previously reported to eliminate the inhibitory TSRC [6]. However, D_0 studies (Fig. 1B) revealed a radioresistant population of these cells also. In contrast to the $CD4^+$ cells, where the D_0 curve flattens out at 4 to 8 Gy, the D_0 curve of the radioresistant $CD8^+$ cells is sloped, indicating a continued degree of radiosensitivity in this resistant fraction. Hence, the populations of residual regulatory cells potentially affecting the hematopoietic system of an irradiated animal vary significantly depending on the radiation dose given. At doses below 4 Gy, the proportion of each of the regulatory cell types examined in this study decreased as the dose changed. Above 4 Gy, the residual $CD4^+$ cell population remained constant while the $CD8^+$ cell content continued to decrease as the radiation dose increased. However, the importance of the changes in the numbers of $CD8^+$ cells at high doses may be ameliorated somewhat by the fact that the overall number of these cells was extremely small after irradiation (Figs. 2 and 3).

Huiskamp et al. [22] previously determined the radiosensitivity of thymocyte subsets using a panel of antibodies that included the CD8 cell marker. The D_0 curve for $CD8^+$ cells, as well as those for each of the other subsets, was biphasic and hence apparently comparable to the results reported in this paper. Although both studies report differences in radiosensitivities based on phenotype, direct comparison of the two sets of data is difficult. This difficulty arises primarily from Huiskamp et al.'s use of cytofluorometry with only one fluorochrome, which cannot distinguish between single-positive $CD8^+$ cells and double-positive $CD4^+/CD8^+$ cells. Consequently, their D_0 curve for the $CD8^+$ cells represents a composite of the radiosensitivity of these two types of cells. Our Figure 1 shows that the D_0 curves for these two populations are markedly different. Resolution of $CD8^+$ and $CD4^+/CD8^+$ as well as identification of $CD4^+$ cells requires the use of two-color analysis with CD8 and CD4 antibodies labeled with different fluorochromes. Comparison of the results between Huiskamp's studies and ours is further impeded not only by their use of fission neutron and X-ray irradiation as opposed to our use of ^{60}Co irradiation, but also by their determination of postirradiation cell survival on days 2 and 5 vs. the 24-hour evaluation time used in our studies. However, both series of studies illustrate a selective survival of phenotypically mature thymocytes following radiation exposure.

We also evaluated the radiosensitivities of splenic lymphocytes and found effects similar to those found in the thymus (Table 1). The effect of radiation on splenic $CD4^+$ cells was similar to that found in the thymus (Table 1). Most of the $CD4^+$ cells were eliminated, but a radioresistant population was readily apparent. Approximately 40% of the splenic $CD4^+$ cells remained after irradiation, as opposed to 13% in the thymus. This may reflect differences in the sensitivity noted between newly formed virgin T cells from the thymus and antigen-experienced T cells found in the peripheral lymphoid organs [9]. The extensive splenic necrosis seen after irradiation, along with the greater heterogeneity of cell types in the spleen as compared to the thymus, may have contributed to the variability seen in the splenic $CD4^+$ cell counts. This variability prevented precise D_0 determinations for the $CD4^+$ spleen cells, but both sensitive and resistant populations were evident (data not shown). Seven days after irradiation, when the variability was greatly reduced, the increased proportion of $CD4^+$ cells in the residual spleen cell population was readily detectable (Fig. 3).

Neither the $CD4^+/CD8^+$ cells nor the $CD4^-/CD8^-$ cells appear to be viable candidates for either the helper or suppressor TSRC. Although $CD4^+/CD8^+$ cells are radiosensitive like the suppressor TSRC, they are restricted to the thymus, while both types of TSRC are found in the bone marrow, spleen, and peripheral blood [3]. $CD4^-/CD8^-$ cells are radioresistant like the helper TSRC but they lack the characteristic peripheral T cell markers.

In the radiation recovery studies presented here, we employed a high sublethal dose of ^{60}Co radiation to induce hematopoietic depletion, which, however, is followed by vigorous recovery initiated from endogenous stem and progenitor cells that survive the irradiation (Fig. 4). The proliferation and differentiation of such stem and progenitor cells are known to be regulated by a variety of cytokines produced by the cells that constitute the hematopoietic environment. These cells include both the stromal elements that make up the framework of the hematopoietic organs and the monocytoid accessory cells from peripheral blood, such as T lymphocytes and macrophages [23]. Our results show that when CFU-S and GM-CFC become detectable in the spleen around day 14, they reside in an environment enriched with $CD4^+$ cells. The finding that in vivo antibody-mediated ablation of $CD4^+$ cells prior to irradiation reduces bone marrow CFU-S and GM-CFC suggests a role for $CD4^+$ cells in hematopoietic recovery [24]. Immunological studies have shown that $CD4^+$ cells produce extensive quantities of a wide range of cytokines [25] such as granulocyte, granulocyte-macrophage, and macrophage colony-stimulating factors (G-CSF, GM-CSF, and M-CSF), interleukin-3 (IL-3), and IL-6, all of which are known to be stimulators of hematopoiesis [26]. Recently many of these cytokines have been used to stimulate hematopoietic recovery in irradiated animals [27–32]. If the residual radioresistant $CD4^+$ cells reported in these experiments produce cytokines, either as a direct result of radiation injury or because of subsequent antigenic stimulation, they may contribute to hematopoietic recovery as proposed by Pantel and Nakeff [33]. Whether these cells are identical to the helper TSRC reported by Sharkis et al. [2] is not definite, but certainly they exhibit similar characteristics based on radiosensitivity. Since these cells are readily identifiable after exposure to radiation, they should be easy to isolate and evaluate for their ability to produce cytokines relevant to hematopoiesis. Studies designed to resolve this issue are currently under way.

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